[0001] **NON-INVASIVE PRENATAL DIAGNOSIS**

[0002] CROSS REFERENCE TO RELATED APPLICATIONS

[0003]   This application is a continuation of U.S. Application Serial No. 09/380,696, now U.S. Pat. 6258540 having a §102(e) date of November 29, 1999, which is a §371 national stage of PCT Application No. PCT/GB 98/00690, Filed March 4, 1998.

[0004] BACKGROUND OF THE INVENTION

[0005]   This invention relates to prenatal detection methods using non-invasive techniques. In particular, it relates to prenatal diagnosis by detecting fetal nucleic acids in serum or plasma from a maternal blood sample.

[0006]   Conventional prenatal screening methods for detecting fetal abnormalities and for sex determination traditionally use fetal samples derived by invasive techniques such as amniocentesis and chorionic villus sampling. These techniques require careful handling and present a degree of risk to the mother and to the pregnancy.

[0007]   More recently, techniques have been devised for predicting abnormalities in the fetus and possible complications in pregnancy, which use maternal blood or serum samples. Three markers commonly used include alpha-foetoprotein (AFP - of fetal origin), human chorionic gonadotrophin (hCG) and estriol, for screening for Down's Syndrome and neural tube defects. Maternal serum is also currently used for biochemical screening for chromosomal aneuploidies and neural tube defects. The passage of nucleated cells between the mother and fetus is now a well recognized phenomenon (Lo et al. 1989; Lo et al. 1996). The use of fetal cells in maternal blood for non-invasive prenatal diagnosis (Simpson and Elias 1993) avoids the risks associated with conventional invasive techniques. WO 91/08304

describes prenatal genetic determination using fetal DNA obtained from fetal cells in the maternal blood. Considerable advances have been made in the enrichment and isolation of fetal cells for analysis (Simpson and Elias 1993; Cheung et al 1996). However, these techniques are time-consuming or require expensive equipment.

[0008] Recently, there has been interest in the use of plasma or serum-derived DNA for molecular diagnosis (Mulcahy et al 1996). In particular, it has been demonstrated that tumor DNA can be detected by the polymerase chain reaction (PCR) in the plasma or serum of some patients (Chen et al 1996; Nawroz et al 1996).

[0009] GB 2 299 166 describes non-invasive cancer diagnosis by detection of K-ras and N-ras gene mutations using PCR-based techniques.

[0010] SUMMARY AND OBJECTS OF THE INVENTION

[0011] It has now been discovered that fetal DNA is detectable in maternal serum or plasma samples. This is a surprising and unexpected finding; maternal plasma is the very material that is routinely discarded by investigators studying noninvasive prenatal diagnosis using fetal cells in maternal blood. The detection rate is much higher using serum or plasma than using nucleated blood cell DNA extracted from a comparable volume of whole blood, suggesting that there is enrichment of fetal DNA in maternal plasma and serum. In fact, the concentration of fetal DNA in maternal plasma expressed as a % of total DNA has been measured as from 0.39% (the lowest concentration measured in early pregnancy), to as high as 11.4% (in late pregnancy), compared to ratios of generally around 0.001 % and up to only 0.025% for cellular fractions (Hamada et al 1993). It is important that fetal DNA is found in maternal plasma as well as serum because this indicates that the DNA is not an artefact of the clotting process.

[0012] This invention provides a detection method performed on a maternal serum or plasma sample from a pregnant female, which method comprises detecting the presence of

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a nucleic acid of fetal origin in the sample. The invention thus provides a method for prenatal diagnosis.

[0013]   The term "prenatal diagnosis" as used herein covers determination of any maternal or fetal condition or characteristic which is related to either the fetal DNA itself or to the quantity or quality of the fetal DNA in the maternal serum or plasma. Included are sex determination, and detection of fetal abnormalities which may be for example chromosomal aneuploidies or simple mutations. Also included is detection and monitoring of pregnancy-associated conditions such as pre-eclampsia which result in higher or lower than normal amounts of fetal DNA being present in the maternal serum or plasma. The nucleic acid detected in the method according to the invention may be of a type other than DNA e.g. mRNA.

[0014]   The maternal serum or plasma sample is derived from the maternal blood. As little as $10\mu l$ of serum or plasma can be used. However it may be preferable to employ larger samples in order to increase accuracy. The volume of the sample required may be dependent upon the condition or characteristic being detected. In any case, the volume of maternal blood which needs to be taken is small.

[0015]   The preparation of serum or plasma from the maternal blood sample is carried out by standard techniques. The serum or plasma is normally then subjected to a nucleic acid extraction process. Suitable methods include the methods described herein in the examples, and variations of those methods. Possible alternatives include the controlled heating method described by Frickhofen and Young (1991). Another suitable serum and plasma extraction method is proteinase K treatment followed by phenol/chloroform extraction. Serum and plasma nucleic acid extraction methods allowing the purification of DNA or RNA from larger volumes of maternal sample increase the amount of fetal nucleic acid material for analysis and thus improve the accuracy. A sequence-based enrichment method could also be used on the maternal serum or plasma to specifically enrich for fetal nucleic acid sequences.

[0016] An amplification of fetal DNA sequences in the sample is normally carried out. Standard nucleic acid amplification systems can be used, including PCR, the ligase chain reaction, nucleic acid sequence based amplification (NASBA), branched DNA methods, and so on. Preferred amplification methods involve PCR.

[0017] The method according to the invention may be particularly useful for sex determination which may be carried out by detecting the presence of a Y chromosome. It is demonstrated herein that using only $10\mu l$ of plasma or serum a detection rate of 80% for plasma and 70% for serum can be achieved. The use of less than 1 ml of maternal plasma or serum has been shown to give a 100% accurate detection rate.

[0018] The method according to the invention can be applied to the detection of any paternally-inherited sequences which are not possessed by the mother and which may be for example genes which confer a disease phenotype in the fetus. Examples include:

[0019] a) Fetal rhesus D status determination in rhesus negative mothers (Lo et al 1993). This is possible because rhesus D positive individuals possess the rhesus D gene which is absent in rhesus D negative individuals. Therefore, the detection of rhesus D gene sequences in the plasma and serum of a rhesus D negative mother is indicative of the presence of a rhesus D positive fetus. This approach may also be applied to the detection of fetal rhesus D mRNA in maternal plasma and serum.

[0020] b) Haemoglobinopathies (Camaschella et al 1990). Over 450 different mutations in the beta-globin gene have been known to cause betathalassaemia. Provided that the father and mother carry different mutations, the paternal mutation can be used as an amplification target on maternal plasma and serum, so as to assess the risk that the fetus may be affected.

[0021]   c)   Paternally-inherited DNA polymorphisms or mutations. Paternally inherited DNA polymorphisms or mutations present on either a Y or a non-Y chromosome, can be detected in maternal plasma and serum to assess the risk of the fetus being affected by a particular disease by linkage analysis. Furthermore, this type of analysis can also be used to ascertain the presence of fetal nucleic acid in a particular maternal plasma or serum sample, prior to diagnostic analysis such as sex determination. This application will require the prior genotyping of the father and mother using a panel of polymorphic markers and then an allele for detection will be chosen which is present in the father, but is absent in the mother.

[0022]   The plasma or serum-based non-invasive prenatal diagnosis method according to the invention can be applied to screening for Down's Syndrome and other chromosomal aneuploidies. Two possible ways in which this might be done are as follows:

[0023]   a)   It has been found that in pregnancy involving fetuses with chromosomal aneuploidies e.g. Down's Syndrome, the level of fetal cells circulating in maternal blood is higher than in pregnancies involving normal fetuses (Bianchi et al 1996). Following the surprising discovery disclosed herein that fetal DNA is present in maternal plasma and serum, it has also been demonstrated that the level of fetal DNA in maternal plasma and serum is higher in pregnancies where the fetus has a chromosomal aneuploidy than in normal pregnancies. Quantitative detection of fetal nucleic acid in the maternal plasma or serum e.g. a quantitative PCR assay, can be used to screen pregnant women for chromosomal aneuploidies.

[0024]   b)   A second method involves the quantitation of fetal DNA markers on different chromosomes. For example, for a fetus affected by Down's Syndrome the absolute quantity of fetal chromosomal 21-derived DNA will always be greater than that from the other chromosomes. The recent development of very accurate quantitative PCR techniques, such as real time quantitative PCR (Heid et al 1996) facilitates this type of analysis.

[0025]   Another application of the accurate quantitation of fetal nucleic acid levels in the maternal serum or plasma is in the molecular monitoring of certain placental pathologies, such as pre-eclampsia. The concentration of fetal DNA in maternal serum and plasma is elevated in pre-eclampsia. This is probably due to the placental damage which occurs.

[0026]   It is anticipated that it will be possible to incorporate the nucleic acid-based diagnosis methods described herein into existing prenatal screening programed. Sex determination has successfully been performed on pregnancies from 7 to 40 weeks of gestation.

[0027]   BRIEF DESCRIPTION OF THE DRAWINGS

[0028]   In the attached figures:

[0029]   Figure 1 shows increased fetal DNA in aneuploid pregnancies compared to control pregnancies;

[0030]   Figure 2 shows increased fetal DNA in pre-eclampsia compared to control pregnancies;

[0031]   Figures 3A and 3B show an amplification curve and threshold cycle for real time quantitative PCR;

[0032]   Figures 4A-4L show fetal DNA concentrations in maternal samples for a number of subjects at different stages of gestation.

[0033] DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0034] The invention will now be illustrated in the following Examples, which do not in any way limit the scope of the invention.

[0035] **EXAMPLES**

[0036] <u>**Example 1**</u>

[0037] **Analysis of fetal DNA for sex determination**

[0038] **Patients**

[0039] Pregnant women attending the Nuffield Department of Obstetrics & Gynaecology, John Radcliffe Hospital, Oxford were recruited prior to amniocentesis or delivery. Ethics approval of the project was obtained from the Central Oxfordshire Research Ethics Committee. Informed consent was sought in each case. Five to ten ml of maternal peripheral blood was collected into an EDTA and a plain tube. For women undergoing amniocentesis, maternal blood was always collected prior to the procedure and 10 ml of amniotic fluid was also collected for fetal sex determination. For women recruited just prior to delivery, fetal sex was noted at the time of delivery. Control blood samples were also obtained from 10 nonpregnant female subjects and further sample processing was as for specimens obtained from pregnant individuals.

[0040] **Sample preparation**

[0041] Maternal blood samples were processed between 1 to 3 hours following venesection. Blood samples were centrifuged at 3000g and plasma and serum were carefully removed from the EDTA-containing and plain tubes, respectively, and transferred into plain polypropylene tubes. Great care was taken to ensure that the buffy coat or the blood clot was undisturbed when plasma or serum samples, respectively, were removed. Following removal of the plasma samples, the red cell pellet and buffy coat were saved for DNA extraction

using a Nucleon DNA extraction kit (Scotlabs, Strathclyde, Scotland, U.K.). The plasma and serum samples were then subjected to a second centrifugation at 3000g and the recentrifuged plasma and serum samples were collected into fresh polypropylene tubes. The samples were stored at -20°C until further processing.

[0042]   **DNA extraction from plasma and serum samples**

[0043]   Plasma and serum samples were processed for PCR using a modification of the method of Emanuel and Pestka (1993). In brief, 200 μl of plasma or serum was put into a 0.5ml eppendorf tube. The sample was then heated at 99°C for 5 minutes on a heat block. The heated sample was then centrifuged at maximum speed using a microcentrifuge. The clear supernatant was then collected and 10 μl was used for PCR.

[0044]   **DNA extraction from amniotic fluid**

[0045]   The amniotic fluid samples were processed for PCR using the method of Rebello et al (1991). One hundred μl of amniotic fluid was transferred into a 0.5 ml eppendorf tube and mixed with an equal volume of 10% Chelex-100 (Bio-Rad). Following the addition of 20 μl of mineral oil to prevent evaporation, the tube was incubated at 56°C for 30 minutes on a heat block. Then, the tube was vortexed briefly and incubated at 99°C for 20 minutes. The treated amniotic fluid was stored at 4°C until PCR and 10 μl was used in a 100 μl reaction.

[0046]   **Polymerase chain reaction (PCR)**

[0047]   The polymerase chain reaction (PCR) was carried out essentially as described (Saiki et al 1988) using reagents obtained from a GeneAmp DNA Amplification Kit (Perkin Elmer, Foster City, CA, USA). The detection of Y-specific fetal sequence from maternal plasma, serum and cellular DNA was carried out as described using primers Y1.7 and Y1.8, designed to amplify a single copy Y sequence (DYS14) (Lo et al 1990). The sequence of Y1.7 is 5' CAT CCA GAG CGT CCC TGG CTT 3' [SEQ ID NO: 1] and that of Y1.8 is 5' CTT TCC ACA GCC ACA TTT GTC 3' [SEQ ID NO: 2]. The Y-specific product was 198

bp. Sixty cycles of Hot Start PCR using Ampliwax technology were used on 10 μl of maternal plasma or serum or 100 ng of maternal nucleated blood cell DNA (denaturation step of 94°C 1 minute and a combined reannealing/extension step of 57°C 1 minute). Forty cycles were used for amplification of amniotic fluid. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. PCR results were scored before the fetal sex was revealed to the investigator.

[0048]   **Results**

[0049]   Sensitivity of PCR assay

[0050]   Serial dilutions of male genomic DNA in 1 μg of female genomic DNA were performed and amplified by the Y-PCR system using 60 cycles of amplification. Positive signals were detected up to the 100,000 dilution, i.e., approximately the equivalent of a single male cell.

[0051]   Amplification of fetal DNA sequence from maternal plasma and serum

[0052]   Maternal plasma and serum samples were collected from 43 pregnant women with gestational ages from 12 to 40 weeks. There were 30 male fetuses and 13 female fetuses. Of the 30 women bearing male fetuses, Y-positive signals were detected in 24 plasma samples and 21 serum samples, when 10 μl of the respective samples was used for PCR. When nucleated blood cell DNA was used for Y-PCR, positive signals were only detected in 5 of the 30 cases. None of the 13 women bearing female fetuses and none of the 10 non-pregnant female controls resulted in a positive Y signal when either plasma, serum or cellular DNA was amplified. Accuracy of this technique, even with serum/plasma samples of only 10 μl, is thus very high and most importantly it is high enough to be useful. It will be evident that accuracy can be improved to 100% or close to 100%, for example by using a larger volume of serum or plasma.

[0053]   **Example 2**

[0054]   **Quantitative analysis of fetal DNA in maternal serum in aneuploid pregnancies**

[0055]   The prenatal screening and diagnosis of fetal chromosomal aneuploidies is an important part of modern obstetrical care. Due to the risks associated with invasive procedures such as amniocentesis and the impracticability of performing screening with invasive methods, much effort has been devoted to the development of non-invasive screening methods for fetal chromosomal aneuploidies. The two main non-invasive methods which have been developed are maternal serum biochemical screening and ultrasound examination for nuchal translucency. These methods are both associated with significant false-positive and false-negative rates.

[0056]   The demonstration of fetal nucleated cells in maternal circulation offers a new source of fetal material for the noninvasive diagnosis of fetal chromosomal aneuploidies (Simpson et al 1993). By the use of fetal nucleated cell enrichment protocols, several groups have reported the detection of aneupioid fetal nucleated cells isolated from maternal blood (Elias et al 1992; Bianchi et al 1992). Recently, it has been demonstrated that there is increased fetal nucleated cell number in maternal circulation when the fetus is suffering from a chromosomal aneuploidy (Bianchi et al 1997).

[0057]   **Patients samples**

[0058]   Blood samples from pregnant women undergoing prenatal testing were collected prior to any invasive procedure. The fetal karyotype was confirmed by cytogenetic analysis of amniotic fluid or chorionic villas samples. Approval was obtained from the Research Ethics Committee of The Chinese University of Hong Kong. Blood samples were collected into plain tubes. Following blood clotting, the samples were centrifuged at 3000 g, and serum were carefully removed and transferred into plain polypropylene tubes. The samples were stored at -70 °C or -20 °C until further processing.

[0059]   **DNA extraction from plasma and serum samples**

[0060]   DNA from serum samples were extracted using a QIAamp Blood Kit (Qiagen, Hilden, Germany) using the "blood and body fluid protocol" as recommended by the manufacturer (Chen et al 1996). Four hundred to 800 μl of plasma/serum sample was used for DNA extraction per column. The exact amount used was documented to enable the calculation of target DNA concentration.

[0061]   **Real time quantitative PCR**

[0062]   Theoretical and practical aspects of real time quantitative PCR were previously described by Head et al (1996). Real time quantitative PCR analysis was performed using a PE Applied Biosystems 7700 Sequence Detector (Foster City, CA, U.S.A.) which is essentially a combined thermal cycler/fluorescence detector with the ability to monitor the progress of individual PCR reactions optically. The amplification and product reporting system used is based on the 5' nuclease assay (Holland et al 1991) (the TaqMan assay as marketed by Perkin-Elmer). In this system, apart from the two amplification primers as in conventional PCR, a dual labeled fluorogenic hybridization probe is also included (Lee et al 1993; Livak et al 1995). One fluorescent dye serves as a reporter (FAM, i.e., 6-carboxyfluorescein) and its emission spectra is quenched by a second fluorescent dye (TAMRA, i.e., 6-carboxy-tetramethylrhodamine). During the extension phase of PCR, the 5' to 3'-exonuclease activity of the Taq DNA polymerase cleaves the reporter from the probe thus releasing it from the quencher, resulting in an increase in fluorescent emission at 518 nm. The PE Applied Biosystems 7700 Sequence Detector is able to measure the fluorescent spectra of the 96 amplification wells continuously during DNA amplification and the data are captured onto a Macintosh computer (Apple Computer, Cupertino, CA, U.S.A.).

[0063]   The SRY TaqMan system consisted of the amplification primers SRY-109F, 5'-TGG CGA TTA AGT CAA ATT CGC-3' [SEQ ID N0:3]; SRY-245R, 5'-CCC CCT AGT ACC CTG ACA ATG TAT T-3' [SEQ ID N0:4]; and a dual labeled fluorescent TaqMan probe SRY-142T, 5'(FAM)AGC AGT AGA GCA GTC AGG GAG GCA GA(TAMRA)-3'

[SEQ ID NO: 5]. Primer/probe combinations were designed using the Primer Express software (Perkin-Elmer, Foster City, CA, U.S.A.). Sequence data for the SRY gene were obtained from the GenBank Sequence Database (accession number L08063).

[0064] TaqMan amplification reactions were set up in a reaction volume of 50 μl using components (except TaqMan probe and amplification primers) supplied in a TaqMan PCR Core Reagent Kit (Perkin-Elmer, Foster City, CA, U.S.A.). The SRY TaqMan probe were custom-synthesized by PE Applied Biosystems. PCR primers were synthesized by Life Technologies (Gaithersburg, MD, U.S.A.). Each reaction contained 5 μl of 10X buffer A, 300 nM of each amplification primers, 100 nM of the SRY TaqMan probe, 4 mM $MgCl_2$, 200 μM each of dATP, dCTP and dGTP, 400 μM dUTP, 1.25 units of AmpliTaq Gold and 0.5 unit AmpErase uracil N-glycosylase. Five to ten μl of the extracted serum DNA was used for amplification. The exact amount used was recorded for subsequent concentration calculation. DNA amplifications were carried out in 96-well reaction plates that were frosted by the manufacturer to prevent light reflection and were closed using caps designed to prevent light scattering (Perkin-Elmer, Foster City, CA, U.S.A.). Each sample was analyzed in duplicate. A calibration curve was run in parallel and in duplicate with each analysis. The conversion factor of 6.6 pg of DNA per cell was used for expressing the results as copy numbers.

[0065] Thermal cycling was initiated with a 2-minute incubation at 50 °C for the uracil N-glycosylase to act, followed by a first denaturation step of 10 minutes at 95 °C. Then, 40 cycles of 95 °C for 15 s and 60°C for 1 minute were carried out.

[0066] Amplification data collected by the 7700 Sequence Detector and stored in the Macintosh computer were then analyzed using the Sequence Detection System (SDS) software developed by PE Applied Biosystems. The mean quantity of each duplicate was used for further concentration calculation. The concentration expressed in copies/ml was calculated using the following equation:

[0067]
$$C = Q \times \frac{V_{DNA}}{V_{PCR}} \times \frac{1}{V_{ext}}$$

[0068]   where C = target concentration in plasma or serum (copies/ml);

[0069]   Q = target quantity (copies) determined by sequence detector in a PCR;

[0070] V_{DNA} - total volume of DNA obtained following extraction, typically 50 μl per Qiagen extraction;

[0071] V_{PCR} = volume of DNA solution used for PCR, typically 5-10 μl

[0072] V_{ext} = volume of plasma/serum extracted, typically 400-800 μl

[0073]   **Anti-contamination measures**

[0074]   Strict precautions against PCR contamination were used (Kwok et al 1989). Aerosol-resistant pipette tips were used for all liquid handling. Separate areas were used for the setting up of amplification reactions, the addition of DNA template and the carrying out of amplification reactions. The 7700 Sequence Detector offered an extra level of protection in that its optical detection system obviated the need to reopen the reaction tubes following the completion of the amplification reactions, thus minimizing the possibility of carryover contamination. In addition, the TaqMan assay also included a further level of anticontamination measure in the form of pre-amplification treatment using uracil N-glycosylase which destroyed uracil containing PCR products (Longo et al 1990). Multiple negative water blanks were included in every analysis.

[0075]   **Results**

[0076]   **Development of real time quantitative PCR**

[0077]   To determine the dynamic range of real time quantitative PCR, serial dilutions of male DNA were made in water consisting of the DNA equivalent from 1,000 cells to 1 cell and subjected to analysis by the SRY TaqMan system. The fewer the number of target molecules, the more amplification cycles were needed to produce a certain quantity of reporter molecules. The system was sensitive enough to detect the DNA equivalent from a single target cell.

[0078]   A parameter, termed the threshold cycle (C_T) could be defined which was set at 10 standard deviations above the mean base-line fluorescence calculated from cycles 1 to 15 and was proportional to the starting target copy number used for amplification (Held et al 1996). A plot of the threshold cycle (C_T) against the input target quantity, with the latter plotted on a common log scale, demonstrated the large dynamic range and accuracy of real time quantitative PCR.

[0079]   The real time quantitative SRY system was insensitive to the existence of background female DNA from 0 to 12,800 female genomeequivalents. This greatly simplified the application of this system as separate calibration curves did not have to be constructed for different cases due to the presence of different concentrations of fetal and maternal DNA.

[0080]   **Quantitative analysis of fetal SRY gene from maternal serum from aneuploid and control pregnancies**

[0081]   Real time quantitative SRY PCR was carried out for serum DNA extracted from women bearing aneuploid and normal fetuses. Data from individual cases are plotted in Figure 1. Fetal DNA concentration was higher in aneuploid than control pregnancies (Mann-Whitney U Test, p=0.006).

[0082]   **Discussion**

[0083]   In this study we demonstrate that the concentration of fetal DNA in maternal serum is elevated in aneuploid pregnancies. These results indicate that fetal DNA quantitation has the potential to be used as a new screening marker for fetal chromosomal aneuploidies. A large scale population-based study could be carried out to develop cutoff values for screening purposes. It would also be useful to investigate the correlation of fetal DNA concentration with the other biochemical markers for maternal serum biochemical screening.

[0084] The mechanism(s) by which increased amounts of fetal DNA is liberated into maternal circulation in aneuploid pregnancies require further research. One possibility is related to the increased numbers of fetal nucleated cells which are released into the maternal blood in aneuploid pregnancies (Bianchi et al 1997). Another possible mechanism may be increased cell death or turnover which may be associated with chromosomal aneuploidies.

[0085] **Example 3**

[0086] **Non-invasive prenatal determination of fetal RhD status from plasma of RhD-negative pregnant women**

[0087] **Introduction**

[0088] The rhesus blood group system is important in transfusion and clinical medicine, being involved in hemolytic disease of the newborn, transfusion reactions and autoimmune hemolytic anemia. Despite the widespread use of rhesus immunoglobulin prophylaxis in rhesus D (RhD)negative mothers, rhesus isoimmunisation still occurs. In those cases where the father is heterozygous for RhD gene, there is a 50% chance that the fetus is RhD-positive and 50% chance that the fetus is RhDnegative. The prenatal determination of fetal RhD status in these cases is clinically useful because no further prenatal invasive testing or therapeutic manoeuvres are necessary if the fetus can be shown to be RhD-negative.

[0089] Advances towards this goal have been made possible recently through the cloning of the human RhD gene (Le Van Kim et al 1992) and the demonstration that RhD-negative individuals lack the RhD gene (Colin et al 1991). Prenatal determination of fetal RhD status has been performed using PCR-based techniques on amniotic fluid samples (Bennett et al 1993).

[0090] A number of groups have also investigated the possibility of using fetal cells in maternal blood for the determination of fetal RhD status (Lo et al 1993). The main problem with this approach is that the system is not sufficiently reliable without fetal cell enrichment or isolation procedure as demonstrated by the high false-positive and

false-negative rates on unenriched samples. Fetal cell enrichment or isolation procedures, on the other hand, are tedious and expensive to perform (Geifman-Holtzman et al 1996; Sekizawa et al 1996).

[0091]   Our discovery of the presence of fetal DNA in maternal plasma and serum offers a new approach for non-invasive prenatal diagnosis.

[0092]   **Materials and Methods**

[0093]   **Patients**

[0094]   Pregnant women attending the Nuffield Department of Obstetrics & Gynaecology were recruited with informed consent. Approval of the project was obtained from the Central Oxfordshire Research Ethics Committee. Women in the second trimester of pregnancy were recruited just prior to amniocentesis. Blood samples were collected prior to any invasive procedures. Ten ml of amniotic fluid was also collected for fetal RhD genotyping. Women in the third trimester of pregnancy were recruited just prior to delivery. A sample of cord blood was taken following delivery for the ascertainment of fetal RhD status by serological methods.

[0095]   **Sample preparation**

[0096]   Blood samples were collected into tubes containing EDTA. The samples were centrifuged at 3000 g, and plasma was carefully removed and transferred into plain polypropylene tubes. Great care was taken to ensure that the buffy coat was not disturbed. The buffy coat samples were stored at -20 °C until further processing. The plasma samples were then recentrifuged at 3000 g and plasma was again carefully removed and transferred into a fresh set of plain polypropylene tubes. The samples were stored at -20 °C until further processing.

[0097]   **DNA extraction from plasma and serum samples**

[0098]   DNA from plasma and buffy coat samples were extracted using a QIAamp Blood Kit (Qiagen, Hiiden, Germany) using the "blood and body fluid protocol" as

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recommended by the manufacturer (Cher et al 1996). Eight hundred μl of plasma sample and 200 μl of buffy coat sample was used for DNA extraction per column.

[0099]   **Real time quantitative PCR**

[0100]   Real time quantitative PCR analysis was performed as described in Example 2 with the following modifications.

[0101]   The RhD TaqMan system consisted of the amplification primers RD-A: 5'-CCT CTC ACT GTT GCC TGC ATT-3' [SEQ ID NO: 6]; RD-B: 5'-AGT GCC TGC GCG AAC ATT-3' [SEQ ID NO: 7]; and a dual labelled fluorescent TaqMan probe RD-T, 5'-(FAM)TAC GTG AGA AAC GCT CAT GAC AGC AAA GTC T(TAMRA)-3' [SEQ ID NO: 8]. Primer/probe combinations were designed using the Primer Express software (Perkin-Elmer, Foster City, CA, U.S.A.). Sequence data for the RhD gene were as previously described (Le Van Kim et al 1992).

[0102]   The beta-globin TaqMan system consisted of the amplification primers beta-globin-354F, 5'-GTG CAC CTG ACT CCT GAG GAG A-3' [SEQ ID NO: 9]; beta-globin-455R, 5'-CCT TGA TAC CAA CCT GCC CAG-3' [SEQ ID NO: 10]; and a dual labelled fluorescent TaqMan probe beta-globin-402T, 5'-(FAM)AAG GTG AAC GTG GAT GAA GTT GGT GG(TAMRA)-3' [SEQ ID NO: 11]. Primer/probe combinations were designed using the Primer Express software (Perkin-Elmer, Poster City, CA, U.S.A.). Sequence data were obtained from the GenBank Sequence Database: accession number U01317.

[0103]   **Results**

[0104]   **Development of real time TaqMan PCR**

[0105]   The real time sequence detector is able to measure the fluorescence intensity of the liberated reporter molecules cycle after cycle. A parameter, termed the threshold cycle (C_T), could be defined which was set at 10 standard deviations above the mean base-line fluorescence calculated from cycles 1 to 15 (Held et al 1996). An amplification reaction in

which the fluorescence intensity rises above the threshold during the course of thermal cycling is defined as a positive reaction.

[0106]   To determine the sensitivity of TaqMan PCR, serial dilutions of genomic DNA isolated from a RhD-positive individual were made in water consisting of the DNA equivalent from 1,000 cells to 1 cell and subjected to analysis by the SRY TaqMan system. The fewer the number of target molecules, the more amplification cycles were needed to produce a certain quantity of reporter molecules. The system was sensitive enough to detect the DNA equivalent from a single target cell.

[0107]   **Correlation of serology and genotyping of the RhD-negative women**

[0108]   The 21 pregnant women enrolled in this study were all serologically RhD-negative. Genomic DNA (10 ng) isolated from the buffy coat from each woman was subjected to the RhD TaqMan assay and in each case a negative result was found; thus demonstrating complete correlation between the serology and genotyping.

[0109]   **RhD genotyping from DNA isolated from maternal plasma**

[0110]   DNA extracted from the plasma of the 21 RhD-negative pregnant women were subjected to the RhD TaqMan assay. There was complete correlation between the fetal RhD genotype predicted from maternal plasma analysis and the result obtained from genotyping the amniotic fluid and serological testing of the cord blood (Table 1).

[0111]   As a control for the amplifiability of DNA extracted from maternal plasma, these samples were also subjected to the beta-globin TaqMan assay. In every case, a positive TaqMan signal was generated.

[0112]   **Discussion**

[0113]   In this study we have demonstrated the feasibility of performing non-invasive fetal RhD genotyping from maternal plasma. This represents the first description of single gene diagnosis from maternal plasma. Our results indicate that this form of genotyping is

highly accurate and can potentially be used for clinical diagnosis. This high accuracy is probably the result of the high concentration of fetal DNA in maternal plasma.

[0114]   The rhesus family of polypeptides are encoded by two related genes: the CcEe gene and the RhD gene (Le Van Kim et al 1992; Cherif-Zahar et al 1990). Due to the complexity of the Rh genetic systems, a number of primer sets have been described for RhD genotyping (Bennet et al 1993; Lo et al 1993; Aubin et al 1997). In order to ensure the accuracy of our genotyping system in the study samples, we performed a control genotyping of buffy coat DNA of our patient population. In all cases there was complete correlation between serology and genotype. It is likely that for robust clinical diagnosis, multiple primer sets are preferred. The TaqMan chemistry can easily accommodate the inclusion of multiple primer/probe sets.

[0115]   The correlation between the severity of fetal hemolytic disease and maternal and-D level is an area which required further investigation. It is possible that increased amount of fetal DNA is liberated into the maternal circulation in the presence of increased fetal hemolysis.

[0116]   **Table 1**

[0117]   **RhDd genotyping from plasma from RhD-negative pregnant women**

Case	Fetal RhD genotype	Maternal Plasma RhD TaqMan Signal
1	-	-
2	-	-
3	-	-
4	+	+
5	+	+
6	-	-
7	-	-

8	+	+
9	+	+
10	-	-
11	+	+
12	+	+
13	+	+
14	+	+
15	-	-
16	+	+
17	+	+
18	+	+
19	+	+
20	+	+
21	+	+

[0118]   **Example 4**

[0119]   **Elevation of fetal DNA concentration in maternal serum in pre-eclamptic pregnancies**

[0120]   **Introduction**

[0121]   Pre-eclampsia is an important cause of maternal and fetal mortality and morbidity. Despite much research, the pathogenesis of this condition is still unclear. The disorder is mainly recognized by the concurrence of pregnancy-induced changes which regress after delivery, of which hypertension and proteinuria are the most commonly used clinical criteria. Some investigators have suggested that pre-eclampsia is the result of abnormal trophoblastic implantation, probably mediated by immunological mechanisms. Other investigators have found pathological changes in the spiral arteries in the decidua and myometrium in which partial occlusion by fibrinoid material is one feature.

[0122]   In this Example we use a real time quantitative PCR assay to show the concentration of fetal DNA in the serum of women suffering from pre-eclampsia. Y chromosomal sequences from male fetuses were used as a fetal marker.

[0123]   **Materials and Methods**

[0124]   **Patients**

[0125]   Pregnant women attending the Department of Obstetrics & Gynaecology at the Prince of Wales Hospital, Shatin, Hong Kong and the Nuffield Department of Obstetrics & Gynaecology, Oxford, U.K. were recruited with informed consent. Approval was obtained from the Research Ethics Committee of The Chinese University of Hong Kong and the Central Oxfordshire Research Ethics Committee. Pre-eclampsia was defined as a sustained rise in diastolic blood pressure to 90 mmHg or higher from previously lower values, with new and sustained proteinuria in the absence of urinary tract infection. The control pregnant women were not on medication and had no hypertension or proteinuria (defined as more than a trace on dipstick urinalysis). The pre-eclamptic and control subjects were matched for gestational age.

[0126]   **Sample preparation**

[0127]   Blood samples were collected into plain tubes. Following blood clotting, the samples were centrifuged at 3000 g, and serum were carefully removed and transferred into plain polypropylene tubes. The samples were stored at -70 °C or -20 °C until further processing.

[0128]   **DNA extraction from plasma and serum samples**

[0129]   DNA from serum samples were extracted using a QlAamp t Blood Kit (Qiagen, Hilden, Germany) using the "blood and body fluid protocol" as recommended by the manufacturer (Chen et al 1996). Four hundred to 800 μl of plasma/serum sample was used for DNA extraction per column. The exact amount used was documented to enable the calculation of target DNA concentration.

[0130]   **Real time quantitative PCR**

[0131]   Real time quantitative PCR analysis was performed as described in Example

2.

[0132]   **Results**

[0133]   **Quantitative analysis of fetal SRY gene from maternal serum**

[0134]   Real time quantitative SRY PCR was carried out for serum DNA extracted from pre-eclamptic and control patients. Data from individual cases are plotted in Figure 2. The median fetal DNA concentrations in pre-eclamptic and control pregnancies were 381 copies/ml and 76 copies/ml, respectively. Fetal DNA concentration was higher in pre-eclamptic than control pregnancies (Mann-Whitney U Test, $p < 0.0001$).

[0135]   **Discussion**

[0136]   Our data indicate that the concentration of fetal DNA is higher in pre-eclamptic compared with non-pre-eclamptic pregnancies. These results indicate that fetal DNA concentration measurement in maternal plasma may be used as a new marker for pre-eclampsia. Compared with other markers for pre-eclampsia, fetal DNA measurement is unique in that it is a genetic marker while other markers, such as activin A and inhibin A, are generally hormonal markers. By its nature, a test based on a genetic marker has the advantage that it is completely fetal specific.

[0137]   Further research will be required to investigate whether the level of fetal DNA is related to the severity of pre-eclampsia. Our discovery also opens up research into the potential application of fetal DNA quantitation to predict the occurrence of pre-eclampsia, prior to the development of clinical signs such as hypertension and proteinuria.

[0138]   The mechanism by which increased amounts of fetal DNA is liberated into the circulation of pre-eclamptic women is unclear at present. Possible mechanisms include damage to the placental interface resulting in fetal cell death and the consequent release of fetal DNA into maternal circulation. A second mechanism is due to the increased trafficking

of fetal cells into maternal circulation in pre-eclampsia. Fetal DNA is then liberated following their destruction in the maternal circulation. Future studies correlating the levels of fetal cells and fetal DNA would be necessary to address these issues.

[0139]   **Example 5**

[0140]   **Quantitative analysis of fetal DNA in maternal plasma and serum**

[0141]   **Introduction**

[0142]   We have demonstrated that fetal DNA is present in maternal plasma and serum. Detection of fetal DNA sequences was possible in 80% and 70% of cases using just 10 μl of boiled plasma and serum, respectively (Lo et al 1997).

[0143]   These observations indicate that maternal plasma/serum DNA may be a useful source of material for the non-invasive prenatal diagnosis of certain genetic disorders. To demonstrate that clinical applications are possible, a number of important questions need to be answered. First, fetal DNA in maternal plasma and serum needs to be shown to be present in sufficient quantities for reliable molecular diagnosis to be carried out. Second, data on the variation of fetal DNA in maternal plasma and serum with regard to gestation age is required to determine the applicability of this technology to early prenatal diagnosis.

[0144]   In this Example we have addressed both of these issues by developing a real time quantitative TaqMan polymerase chain reaction (PCR) assay (Heid et al 1996) for measuring the copy numbers of fetal DNA molecules in maternal plasma and serum. This technique permits continuous optical monitoring of the progress of an amplification reaction, giving accurate target quantitation over a wide concentration range. Our data show that fetal DNA is present in maternal plasma and serum at concentrations similar to those achieved by many fetal cell enrichment protocols. We have also investigated the changes of fetal DNA concentration in maternal serum at different gestational ages. Using this plasma or serum-based approach, we show that the reliable detection of fetal DNA is achievable and therefore useful for the non-invasive prenatal diagnosis of selected genetic disorders.

[0145]   **Subjects and Methods**

[0146]   **Patients**

[0147]   Pregnant women attending the Department of Obstetrics & Gynaecology at the Prince of Wales Hospital, Shatin, Hong Kong were recruited with informed consent. Approval was obtained from the Research Ethics Committee of The Chinese University of Hong Kong. For women studied at a single time point, early pregnancy samples were obtained prior to amniocentesis or chorionic villus sampling while late pregnancy samples were collected just prior to delivery. Five to ten ml of maternal peripheral blood was collected each into one tube containing EDTA and one plain tube. Subjects studied at multiple time points were recruited from the *in vitro* fertilization program, prior to conception. Five to ten ml of maternal blood from these subjects was collected into a plain tube at each studied time point. For women undergoing prenatal diagnosis, the sex of the baby was ascertained from cytogenetic results from the amniocentesis or chorionic villus samples. For women recruited just prior to delivery or from the in vitro fertilization program, fetal sex was noted at the time of delivery.

[0148]   **Sample preparation**

[0149]   Blood samples were centrifuged at 3000 g, and plasma and serum were carefully removed from the EDTA-containing and plain tubes, respectively, and transferred into plain polypropylene tubes. Great care was taken to ensure that the buffy coat or the blood clot was undisturbed when plasma or serum samples, respectively, were removed. The plasma and serum samples were recentrifuged at 3000 g and the supernatants were collected into fresh polypropylene tubes. The samples were stored at -20 °C until further processing.

[0150]   **DNA extraction from plasma and serum samples**

[0151]   DNA from plasma and serum samples were extracted using a QIAamp Blood Kit (Qiagen, Hilden, Germany) using the "blood and body fluid protocol" as recommended by the manufacturer (Chen et al 1996). Four hundred to 800 μl of plasma/serum sample was

used for DNA extraction per column. The exact amount used was documented to enable the calculation of target DNA concentration.

[0152]   **Real time quantitative PCR**

[0153]   Real time quantitative PCR analysis was performed as described in Example 2, using the SRY TaqMan system and the betaglobin TaqMan system described in the previous Examples.

[0154]   Identical thermal profile was used for both the SRY and betaglobin TaqMan systems. Thermal cycling was initiated with a 2-minute incubation at 50°C for the uracil N-glycosylase to act, followed by a first denaturation step of 10 minutes at 95 °C. Then, 40 cycles of 95°C for 15 s and 60°C for 1 minute were carried out.

[0155]   **Results**

[0156]   **Development of real time quantitative PCR**

[0157]   To determine the dynamic range of real time quantitative PCR, serial dilutions of male DNA were made in water consisting of the DNA equivalent from 1,000 cells to 1 cell and subjected to analysis by the SRY TaqMan system. Fig. 3A demonstrates that the amplification curve shifted to the right as the input target quantity was reduced. This was expected as reactions with fewer target molecules required more amplification cycles to produce a certain quantity of reporter molecules than reactions with more target molecules. The system was sensitive enough to detect the DNA equivalent from a single target cell.

[0158]   Fig. 3B shows a plot of the threshold cycle (C_T) against the input target quantity, with the latter plotted on a common log scale. The C_T was set at 10 standard deviations above the mean base-line fluorescence calculated from cycles 1 to 15 and was proportional to the starting target copy number used for amplification (Held et al 1996). The linearity of the graph demonstrates the large dynamic range and accuracy of real time quantitative PCR. Similar results were obtained using the beta-globin TaqMan system (results not shown).

[0159]   The real time quantitative SRY system was insensitive to the existence of background female DNA from 0 to 12,800 female genomeequivalents. This greatly simplified the application of this system as within this range, separate calibration curves did not have to be constructed for different cases due to the presence of different concentrations of fetal and maternal DNA.

[0160]   The reproducibility of DNA extraction from plasma and serum using the Qiagen protocol was tested by performing replicate extractions (10 for each case) from plasma and serum samples from normal individuals. These replicate extractions were then subjected to real time quantitative PCR using the beta-globin system. The coefficient of variation (CV) of C_T values of these replicate extractions was 1. 1 %.

[0161]   **Quantitative analysis using the real time beta-globin TaqMan system**

[0162]   The concentration of beta-globin sequences in maternal plasma and serum samples was used as a measure of the total amount of extracted DNA, i.e., maternal and fetal DNA extracted from plasma and serum samples from 50 pregnant women was analyzed using the betaglobin TaqMan system. Twenty-five cases were recruited during the first and second trimesters (gestational age: 11 to 17 weeks) and were denoted as early pregnancy samples in Table 2. The other twenty-five cases were recruited just prior to delivery (gestational age: 37 to 43 weeks) and were denoted as late pregnancy samples in Table 1. The concentrations of beta-globin sequences in maternal plasma and serum are listed in Table 2. These results show that serum contains more DNA than plasma (Wilcoxon Signed Rank Test, p<0.0005), with a mean concentration of serum DNA 14.6 times that of plasma DNA in our studied population. The concentration of beta-globin sequences in maternal plasma from early and late pregnancy samples are compared in Table 2. These data show that the total amount of plasma DNA increases as pregnancy progresses (Mann-Whitney Rank Sum Test, p<0.0005).

[0163]   **Quantitative analysis of fetal SRY gene from maternal plasma and serum**

[0164]   Real time quantitative analysis using the SRY TaqMan system was carried out on DNA extracted from maternal plasma and serum to determine the amount of fetal DNA. Of the 25 early pregnancy samples (gestational age: 11 to 17 weeks), 13 were from women bearing male fetuses and 12 were from women bearing female fetuses. Of the 25 late pregnancy samples (gestational age: 37 to 43 weeks), 14 were from women bearing male fetuses and 11 were from women bearing female fetuses. A positive signal was obtained in each of the 27 women bearing male fetuses and no signal was detected in each of the 23 women bearing female fetuses. Fourteen women had a history of delivering a previous male baby and 5 of these were carrying a female baby in the current studied pregnancy.

[0165]   Quantitative SRY data from the 27 women bearing male fetuses are summarized in Table 3. These data show that the concentrations of fetal DNA in plasma and serum are higher in late gestation than in early gestation (MannWhitney Rank Sum Test, $p < 0.0005$). The mean concentrations of fetal DNA in maternal plasma and serum are 11.5 times and 11.9 times, respectively, higher in late gestation compared with early gestation. The absolute concentrations of fetal DNA in maternal plasma and serum were similar in individual cases. The fractional concentration of fetal DNA in early pregnancy ranges from 0.39% to 11.9% (mean: 3.4%) in plasma and 0.014% to 0.54% (mean: 0.13%) in serum. In late pregnancy, the fraction of fetal DNA ranges from 2.33% to 11.4% (mean: 6.2%) in plasma and 0.032% to 3.97% (mean: 1.0%) in serum.

[0166]   **Sequential follow up of women who conceived by *in vitro* fertilization**

[0167]   Twenty women who conceived by *in vitro* fertilization (IVF) were followed up at pre-conception and at multiple time points during pregnancy. All twenty subjects had singleton pregnancies as determined by ultrasound scanning. Twelve women delivered male babies and the remaining 8 delivered female babies. None of the women carrying male fetuses had a history of pregnancy-associated complications. Subject S-51 (fig. 4I) underwent chorionic villus sampling at 12 weeks. Subjects S-1 and S-56 (figs. 4A and 4K)

had amniocentesis at 16 and 17 weeks, respectively. A total of 163 serum samples from these 20 women were analyzed using the real time quantitative SRY TaqMan system. None of the 65 serum samples from the 8 women bearing female babies gave a positive SRY signal. The concentrations of fetal DNA in the 98 serum samples from women carrying male babies are plotted in Figures 4A-4L.

[0168]   **Discussion**

[0169]   We have developed an accurate real time quantitative PCR system for determining the concentration of fetal DNA in maternal plasma and serum. This system has a number of advantages: (1) a large dynamic range of over 5 orders of magnitude (Held et al 1996); (2) a high throughput and fast turnaround time - 96 samples could be simultaneously amplified and quantified in approximately 2 hours; and (3) the use of a homogeneous amplification/detection system which requires no post-PCR processing and therefore minimizes the risk of carryover contamination.

[0170]   The most important observation in this study is the very high concentration of fetal DNA in maternal plasma and serum. Bianchi et al reported that the average number of fetal cells in maternal blood in normal pregnancies was 19 in 16 ml of maternal blood, i.e., 1.2 cells/ml during the second trimester (Bianchi et al 1997). Therefore, the mean concentration of fetal DNA in maternal plasma and serum is 21.2 (25.4/1.2) and 23.9 (28.7/1.2) times, respectively, higher than that in the cellular fraction of maternal blood at the same gestation. The relative concentration of fetal to total plasma DNA is even higher. Thus, in early pregnancy, fetal DNA in maternal plasma constitutes a mean of 3.4% of the total plasma DNA. The respective figure in-late pregnancy is 6.2%. Hamada et al reported that the frequency of fetal cells in the second trimester was 0.0035% while that in the third trimester was 0.008% (Hamada et al 1993). The fetomaternal ratio is, therefore, 97Sfold and 775-fold higher in maternal plasma than in the cellular fraction at the respective gestational age. Indeed, the fetomaternal ratio in plasma DNA is comparable to that following many fetal

cell enrichment protocols. For example, Bianchi et al reported that following fetal nucleated red cell enrichment using fluorescence activated cell sorting, the resulting fetal cells constituted 0.001 %-5% of the sorted cell populations as determined by quantitative PCR analysis (Bianchi et al 1994). In a similar study using cell sorting and fetal cell detection using fluorescence in situ hybridization, Sohda et al found that on average 4.6% of the sorted cells were of fetal origin (Sohda et al 1997). Maternal plasma, therefore, offers an easily accessible fetal DNA source for prenatal genetic analysis.

[0171]   We have demonstrated that the absolute concentration of fetal DNA in maternal plasma is similar to that in maternal serum. The main difference lies in the presence of a larger quantity of background maternal DNA in serum compared with plasma, possibly due to the liberation of DNA during the clotting process. While this exerts no noticeable effect on the efficiency of fetal DNA detection using the real time TaqMan system, it is possible that with the use of less sensitive methods, e.g., conventional PCR followed by ethidium stained agarose gel electrophoresis, maternal plasma may be preferable to maternal serum for robust fetal DNA detection.

[0172]   The high concentration of fetal DNA in maternal plasma and serum has allowed us to reliably detect the presence of fetal genetic material. Of the 263 serum or plasma samples analyzed in this study, we were able to detect fetal SRY gene in maternal plasma or serum from every subject who was carrying a male baby at the time of venesection. This robust detection rate was obtained using DNA extracted from just 40-80 μl of maternal plasma and serum. This volume represents a 4-8 fold increase over the 10 μl of boiled maternal plasma or serum reported in our previous study (Lo et al 1997) and results in significant improvement in sensitivity. The specificity was preserved as we did not observe amplification signals from samples obtained pre-conception or from subjects carrying a female fetus. From the data obtained thus far, plasma/serum analysis did not appear to be significantly affected by the persistence of fetal cells from previous pregnancies

(Bianchi et al 1996). Thus, we did not obtain any false positive results from women who had carried a previous male baby but who were carrying a female baby at the time of blood sampling for this study.

[0173]   The sequential study on patients undergoing IVF gave a number of important results. First, all of the 12 patients carrying male babies were shown to be negative for SRY sequences in their sera prior to conception. This provided convincing evidence that the SRY sequence detected by the TaqMan assay did indeed originate from the male fetus in the current pregnancy. Second, we were able to detect fetal SRY sequences as early as the 7th week of gestation; thus indicating that fetal genetic analysis in maternal plasma/serum could be used in the first trimester. Third, we showed that fetal DNA concentration increased as pregnancy progressed Figures 4A-4L. This last point was also confirmed by data obtained from women studied at a single time point. Women recruited late in pregnancy had higher fetal DNA concentrations in their plasma and serum (Table 3).

[0174]   In addition to the increase in fetal DNA concentration as pregnancy progresses, our data also indicate that maternal plasma DNA also increases with gestation (Table 2). The biologic basis for this phenomenon is unclear at present. Possible explanations include the increase in size of the fetomaternal interface as gestation progresses and possible reduction in DNA clearance associated with other physiologic changes in pregnancy.

[0175]   For selected disorders, fetal genetic information could be acquired more economically and rapidly from maternal plasma or serum than by using fetal cells isolated from maternal blood. We envisage that fetal DNA analysis in maternal plasma and serum would be most useful in situations where the determination of fetal-derived paternally-inherited polymorphisms/mutations or genes would be helpful in clinical prenatal diagnosis (Lo et al 1994). Examples include fetal sex determination for the prenatal diagnosis of sex-linked disorders, fetal rhesus D status determination in sensitized rhesus negative pregnant women (Lo et al 1993), autosomal dominant disorders in which the father carries

the mutation and autosomal recessive genetic disorders in which the father and mother carry different mutations (Lo et al 1994), e.g., certain hemoglobinopathies (Camaschella et al 1990) and cystic fibrosis. Due to the much reduced maternal background and high fetal DNA concentration in maternal plasma and serum, we predict that this type of analysis would be much more robust compared with their application for detecting unsorted fetal cells in maternal blood. The ability for allelic discrimination (Lee et al 1993; Livak et al 1995) allows the homogeneous TaqMan assay to be used for this purpose. The high throughput and anticontamination capability of this system makes it an attractive candidate for large scale clinical application.

[0176]   Bianchi et al recently reported that fetal cells in maternal blood were increased in aneuploid pregnancies (Bianchi et al 1997) and it has been demonstrated (Example 2) that the fetal DNA concentration in maternal plasma and serum is also elevated in these pregnancies. This provides a new screening test for fetal chromosomal disorders. For this application, fetal DNA quantitation systems can be developed for polymorphic markers outside the Y chromosome so that quantitation can be applied to female fetuses. Autosomal polymorphic systems which may be used for this purpose have already been described (Lo et al 1996). However, fetal cell isolation techniques would still be necessary for a definitive cytogenetic diagnosis. Similarly, fetal cell isolation would also be required for direct mutational analysis of autosomal recessive disorders caused by a single mutation. It is likely that fetal cell isolation and analysis of fetal DNA in maternal plasma/serum would be used as complementary techniques for non-invasive prenatal diagnosis.

[0177]   The biologic basis by which fetal DNA is liberated into maternal plasma remains to be elucidated. It is possible that fetal DNA is released from cell lysis resulting from physical and immunologic damage, or through developmentally associated apoptosis of fetal tissues. It is also likely that increased amounts of fetal DNA may be found in conditions associated with placental damage, such as pre-eclampsia. The real time

quantitative PCR system described here offers a powerful tool to study these unexplored pathophysiologic aspects of fetal DNA in maternal plasma and may improve our understanding of the fetomaternal relationship.

[0178]   **Table 2**

[0179]   **Quantitative analysis of maternal plasma and serum using the beta-globin TaqMan assay**

	Mean (copies/ml)	Median (copies/ml)	Range (copies/ml)
Plasma (Early + Late Pregnancy)	3466	1594	356-31875
Serum (Early + Late Pregnancy)	50651	34688	5813-243750
Plasma (Early Pregnancy)	986	975	356-1856
Plasma (Late Pregnancy)	5945	4313	1125-31875

[0180]   **Table 3**

[0181]   **Quantitation of fetal DNA in maternal plasma and serum: relationship with gestational age**

SRY concentation (copies/ml)

	Early Pregnancy		Late Pregnancy	
	Plasma	Serum	Plasma	Serum
Range	3.3 - 69.4	4.0- 58.1	76.9 - 769	33.8 - 900
Mean	25.4	28.7	292.2	342.1
Median	20.6	19.5	244.0	286.0

[0182]   **Figure Legends**

[0183]   Figure 1. Fetal DNA in maternal serum from women carrying aneuploid and normal fetuses. The control and aneuploid groups are as indicated on the x-axis. The fetal SRY DNA concentrations expressed in copies/ml are plotted on the y-axis.

[0184]   Figure 2. Fetal DNA in maternal serum in pre-eclamptic and non-pre-eclamptic pregnancies. The pre-eclamptic and control groups are as indicated on the x-axis. The fetal SRY DNA concentrations expressed in copies/ml are plotted on the y-axis.

[0185]   Figures 3A and 3B. Real time quantitative PCR. A, Amplification plots obtained using real time quantitative PCR for the SRY gene. Each plot corresponds to a particular input target quantity marked by a corresponding symbol. The x-axis denotes the cycle number of a quantitative PCR reaction. The y-axis denotes the ΔRn which is the fluorescence intensity over the background (Heid et al 1996). B, Plot of the threshold cycle (C_T) against the input target quantity (common log scale). The correlation coefficient is 0.986.

[0186]   Figures 4A-4L. Sequential study of 12 women bearing male fetuses who conceived by *in vitro* fertilization. Each case is denoted by a unique recruitment case number. The x-axis denotes the gestation at which the serum sample was obtained. A gestation age of zero denotes the pre-conception sample. The y-axis denotes the concentration of fetal SRY in maternal serum expressed in copies/ml. The scale has been optimized for the concentration range for each case.

[0187]   **References**

[0188]   **Aubin J T**, Le Van Kim C, Mouro I, et al Specificity and sensitivity of RhD genotyping methods by PCRbased DNA amplification. Br J Haematol 1997; **98**: 356-364.

[0189]   **Bennett P R**, Le Van Kim C, Colin Y, et al Prenatal determination of fetal RhD type by DNA amplification. N Engl J Med 1993; **329**: 607-610.

JAK-PT001.1

[0190]   **Bianchi D W,** Mahr A, Zickwolf GK, Houseal TW, Flint AF, Klinger KW, Detection of fetal cells with 47,XY,+21 karyotype in maternal peripheral blood. Hum Genet 1992; **90**:368-370.

[0191]   **B, Shuber AP**, DeMaria MA, Fougner AC, Klinger KW (1994) Fetal cells in maternal blood: determination of purity and yield by quantitative PCR. Am J Obstet Gynecol **171**: 922-926.

[0192]   **Bianchi D W,** Williams J M, Pelletier C, Klinger K W, Shuber A P. Fetal cell quantitation in maternal blood samples from normal and aneuploid pregnancies. Pediatr Res 1996; **39**: 142A.

[0193]   **Bianchi D W,** Williams J M, Sullivan LM, Hanson FW, Klinger KW, Shuber AP. PCR quantitation of fetal cells in maternal blood in normal and aneuploid pregnancies. Am J Hum Genet 1997; **61**:822-829.

[0194]   **Camaschella C,** Alfarano A, Gottardi E, et al Prenatal diagnosis of fetal hemoglobin Lepore-Boston disease on maternal peripheral blood. Blood 1990; **75**: 2102-106.

[0195]   **Cherif-Zahar B,** Bloy C, Le Van Kim C, et al Molecular cloning and protein structure of a human blood group Rh polypeptide. Proc Natl Acad Sci U S A 1990; **87**: 6243-6247.

[0196]   **Chen X Q,** Stroun M, Magnenat J-L, et al Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nat Med 1996; **2**: 1033-35.

[0197]   **Cheung M C,** Goldberg JD, Kan YW. Prenatal diagnosis of sickle cell anemia and thalassemia by analysis of fetal cells in maternal blood. Nat Genet 1996; **14**: 264-68.

[0198]   **Colin Y,** Cherif-Zahar B, Le Van Kim C, Raynal V, van Huffel V, Cartron JP. Genetic basis of the RhD-positive and RhD-negative blood group polymorphism as determined by Southern analysis. Blood 1991; **78**: 27472752.

[0199]   Elias S, Price J, Dockter M, Wachtel S, Tharapel A, Simpson JL. First trimester prenatal diagnosis of trisomy 21 in fetal cells from maternal blood. Lancet 1992; **340**: 1033.

[0200]   Emanuel S L, Pestka S. Amplification of specific gene products from human serum. GATA 1993; **10**: 144-46.

[0201]   Frickhofen N. & Young N.S. A rapid method of sample preparation for detection of DNA viruses in human serum by polymerise chain reaction. J.Virological Methods 1991; **35**: 65-72.

[0202]   Geifman-Holtzman O, Bernstein IM, Berry SM, et al Petal RhD genotyping in fetal cells flow-sorted from maternal blood. Am. J. Obstet. Gynecol. 1996; **174**: 818-822.

[0203]   Hamada H, Arinami T, Kubo T, Hamaguchi H, Iwasaki H (1993) Fetal nucleated cells in maternal peripheral blood: frequency and relationship to gestational age. Hum Genet **91**: 427432

[0204]   Heid C. A., Stevens J., Livak K.J., Williams P.M. Real time quantitative PCR. Genome Research 1996; **6**: 986-994.

[0205]   Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilising the 5'-3' exonuclease activity of the Thermus aquaticus DNA polymerise. Proc Natl Acad Sci USA 1991; **88**: 7276-7280.

[0206]   Kwok S, Higuchi R. Avoiding false positives with PCR. Nature 1989; **339**: 237-238.

[0207]   Le Van Kim C, Mouro I, Cherif-Zahar B, et al Molecular cloning and primary structure of the human blood group RhD polypeptide. Proc Natl Acad Sci USA 1992; **89**:10925-10929.

[0208]   Lee L G, Connell CR, Bloch W. Alldic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res 1993; **21**: 3761-3766.

[0209]   **Livak K J**, Flood SJ, Marmaro J, Giusti W, Deetz K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl 1995; **4**: 357-362.

[0210]   **Lo Y M D**, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CWG, Wainscoat JS (1997) Presence of fetal DNA in maternal plasma and serum. Lancet **350**: 485487

[0211]   **Lo Y M D**, Fleming KA, Wainscoat JS (1994) Strategies for the detection of autosomal fetal DNA sequence from maternal peripheral blood. Ann NY Acad Sci **731**: 204-213

[0212]   **Lo Y M D**, Patel P, Wainscoat J S, Sampietro M, Gillmer M D G, Fleming K A. Prenatal sex determination by DNA amplification from maternal peripheral blood. Lancet 1989; **2**: 1363-65.

[0213]   **Lo Y M D**, Lo E S F, Watson N, et al Two-way cell traffic between mother and fetus: biologic and clinical implications. Blood 1996; **88**: 4390-95.

[0214]   **Lo Y M D**, Patel P, Sampietro M, Gillmer M D G, Fleming K A, Wainscoat JS. Detection of single-copy fetal DNA sequence from maternal blood. Lancet 1990; **335**: 1463-64.

[0215]   **Lo Y M D**, Bowell P J, Selinger M, et al Prenatal determination of fetal RhD status by analysis of peripheral blood of rhesus negative mothers. Lancet 1993; **341**: 1147-48.

[0216]   **Longo M C**, Berninger MS, Hardey JL. Use of uracil DNA glycosylase to control calTy-over contamination in polymerase chain reactions. Gene 1990; **93**: 125-128.

[0217]   **Mulcahy H E**, Croke DT, Farthing M J G. Cancer and mutant DNA in blood plasma. Lancet 1996; **348**: 628.

[0218]   **Nawroz H**, Koch W, Anker P, Stroun M, Sidransky D. Microsatellite alterations in serum DNA of head and neck cancer patients. Nat Med 1996; **2**: 1035-37.

[0219]   **Rebello M T**, Hackett G, Smith J, et al Extraction of DNA from amniotic fluid cells for the early prenatal diagnosis of genetic disease. Prenat Diagn 1991; **11**: 41-46.

[0220]   **Saiki R K,** Gelfand D H, Stoffel S, et al Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerise. Science 1988; **239**: 487-91.

[0221]   **Sekizawa A**, Watanabe A, T. K, Saito H, Yanaihara T, Sato T. Prenatal diagnosis of the fetal RhD blood type using a single fetal nucleated erythrocyte from maternal blood. Obstet Gynecol 1996; **87**: 501-505.

[0222]   **Simpson J L**, Elias S. Isolating fetal cells from maternal blood: advances in prenatal diagnosis through molecular technology. JAMA 1993; **270**: 2357-61.

[0223]   **Sohda S**, Arinami T, Hamada H, Nakauchi H, Hamaguchi H, The proportion of fetal nucleated red blood cells in maternal blood: estimation by FACS analysis. Prenat Diagn **17**: 743-752.

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